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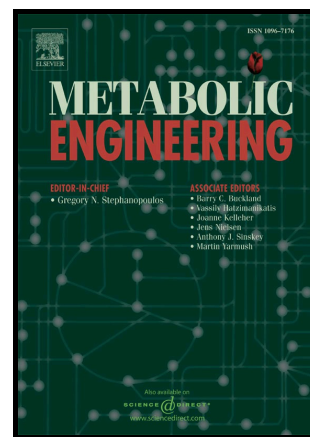
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Alterations in the transcription factors GntR1 and RamA enhance the growth and central metabolism of *Corynebacterium glutamicum*

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## Abstract

Evolution, i.e. the change in heritable characteristics of biological populations over successive generations, has created the diversity of life that exists today. In this study we have harnessed evolution to create faster growing mutants of *Corynebacterium glutamicum*, i.e. to debottleneck growth rate of this highly important industrial workhorse. After approximately 1500 generations of Adaptive Laboratory Evolution (ALE) in defined minimal medium with glucose, we obtained faster growing mutants with specific growth rate as high as 0.64 h<sup>-1</sup> as compared with 0.45 h<sup>-1</sup> for the wild type, and this 42% improvement is the highest reported for *C. glutamicum* to date. By genome resequencing and inverse metabolic engineering, we were able to pinpoint two mutations contributing to most of the growth improvement, and these resided in the transcriptional regulators GntR1 (*gntR1*-E70K) and RamA (*ramA*-A52V). We confirmed that the two

mutations lead to alteration rather than elimination of function, and their introduction in the wild-type background resulted in a specific growth rate of  $0.62 \text{ h}^{-1}$ . The glycolytic and pentose phosphate pathway fluxes had both increased significantly, and a transcriptomic analyses supported this to be associated with increased capacity. Interestingly, the observed fast growth phenotype was not restricted to glucose but was also observed on fructose, sucrose and xylose, however, the effect of the mutations could only be seen in minimal medium, and not rich BHI medium, where growth was already fast. We also found that the mutations could improve the performance of resting cells, under oxygen-deprived conditions, where an increase in sugar consumption rate of around 30% could be achieved. In conclusion, we have demonstrated that it is feasible to reprogram *C. glutamicum* into growing faster and thus enhance its industrial potential.

**Keywords:** *Corynebacterium glutamicum*; adaptive evolution; fast growth; genomic analysis; GntR1; RamA

## Introduction

The specific growth rate is a key parameter, which needs to be considered when assessing the suitability of a particular microorganism for industrial use. For growth-coupled production processes, fast growth is an obvious advantage (Feist et al., 2010; Grünberger et al., 2013; Liu et al., 2016a, 2017), but also in general fast growth is desirable as it allows for a high cell density and high productivity to be attained more rapidly. Finally a fast growth phenotype is desirable to compensate for the reduction in growth rate often seen when harnessing the metabolic machinery for production (Lee and Kim, 2015; Liu et al., 2016b, c). Fast growing organism do exist, however, they are not always suited for industrial production, e.g. due to safety reasons, higher nutritional requirements, etc, and sometimes a better option may be to improve growth properties a candidate more suited to industrial production. Adaptive laboratory evolution (ALE), in combination with various other tools, has become a powerful approach for studying how microorganisms adapt genetically to improve fitness, over time, to certain conditions or changes in environmental parameters (Mahr et al., 2015; Oide et al., 2015; Takeno et al., 2013). A classic example is the work of Richard Lenski and co-workers, who studied adaptation of *Escherichia coli* to growth in a glucose-limited minimal medium over 40,000 generations (Barrick et al., 2009). ALE has also been demonstrated to be extremely useful for optimizing

properties of microorganisms, e.g. metabolic imbalances, inhibitor tolerance, thermal tolerance and ability to use certain carbon (Caspeta et al., 2014; Cheng et al., 2014; Hong et al., 2011; Ibarra et al., 2002; Jakob et al., 2007; Long et al., 2017)

One important reason that has stimulated the use of ALE in recent years is the development of reliable sequencing technologies, which has simplified the task of identifying the correlations between phenotype and genotype (Albert et al., 2005; Dettman et al., 2012; Herring et al., 2006). Cheap and fast access to genome sequences of adapted mutants has made it possible to apply inverse metabolic engineering for creating superior cell factories devoid of the many mutations that inevitably are introduced in the course of ALE (Bailey et al., 1996; Jin et al., 2005). In ALE, beneficial mutations normally emerge after hundreds to thousands of generations, depending on microbe and cultivation conditions (James Winkler, Luis H. Reyes, 2013). However, under some conditions, e.g. during starvation, certain bacteria have been reported to mutate more rapidly, which increases the efficiency of ALE for obtaining useful mutants (Kim et al., 2001).

The actinobacterium *Corynebacterium glutamicum*, is an important and established industrial workhorse due to its robustness and metabolic flexibility, and this organism has been engineered into producing a broad range of useful compounds, such as amino acids, organic acids, alcohols and even non-natural compounds (Becker and Wittmann, 2015, 2012). *C. glutamicum* has quite simple growth requirements and grows well in chemically defined minimal medium containing a suitable carbon source. Various kinds of carbohydrates, e.g. glucose, fructose, and sucrose, or organic acids like acetate, pyruvate and lactate can readily be used by *C. glutamicum* (Ikeda, 2012). What appears to be highly unusual for this bacterium is that it does not appear to have traditional catabolite control repression (CCR) mechanisms, and thus different common carbon substrates can be metabolized simultaneously (Moon et al., 2007).

Various defined media containing glucose have been described for *C. glutamicum*, optimized in terms of growth rate over the past decades, e.g. MM (Liebl et al., 1989), CGXII (Keilhauer et al., 1993) and similar media (Bäumchen et al., 2007; Kind et al., 2010). Recently, Noack and colleagues achieved the fastest growth ( $0.61 \text{ h}^{-1}$ ) ever reported for *C. glutamicum* in glucose minimal medium at the single cell level in a microfluidic perfusion system (Grünberger et al., 2013). Subsequent work revealed that the cause for the fast growth was due to co-metabolism of protocatechuic acid (PCA) and glucose (Unthan et al., 2014). These

results indicate that there are bottlenecks controlling how fast *C. glutamicum* can grow on glucose, and this is also supported by the finding that sucrose supports an even faster growth than glucose (Wang et al., 2016). In *C. glutamicum* several regulators are involved in governing central metabolism. The DeoR-type regulator SugR is considered to be a repressor of several PTS genes, however it has been found that its inactivation only has a marginal effect on the phenotype (Engels and Wendisch, 2007). Yukawa et al., demonstrated, by over-expressing several glycolytic enzymes, that the glycolytic flux could be enhanced, however, only for oxygen deprived resting cells (non-growing), and not under normal aerobic growth conditions (Yamamoto et al., 2012). In our previous studies we found that by perturbing the fructose operon, we could increase the growth rate of *C. glutamicum*, on glucose, by 10% and we found that this increase in growth rate correlated with an accumulation of intracellular fructose-1-phosphate (Wang et al., 2016). Besides these studies, there have been relatively few efforts focusing on finding factors controlling glucose metabolism in *C. glutamicum*, which makes it an interesting topic to study, in particular in the light of the potential implications it could have for bio-based production using this organism.

The objective of the current study is thus to shed additional light on factors controlling the growth and metabolism of carbohydrates. First we adapt the wild-type *C. glutamicum* strain ATCC 13032 on glucose minimum medium for more than 1500 generations and isolate several faster growing mutants. We carefully characterize one of these mutants through growth experiments, genome sequencing, and transcriptome analysis. Single mutations, contributing to most of the faster growth phenotype are identified in the two transcriptional regulators GntR1 and RamA, and we demonstrate that these mutations result in faster metabolism of glucose and other sugars, both by growing and resting cells.

## Material and methods

### Bacterial strains and medium.

All bacterial strains and plasmids used in this study are listed in Supplementary Table 1. For molecular cloning, *E. coli* strains were aerobically grown at 37°C in Luria-Bertani (LB) broth, and *C. glutamicum*

strains were aerobically grown at 30°C in BHI broth (Sigma, Prod. No.53286). When appropriate, the solid medium was supplemented with kanamycin (50 µg/ml for *E. coli*, 25 µg/ml for *C. glutamicum*), or 10% sucrose. For physiological characterization of *C. glutamicum* strains, a defined minimal medium (MM) was used which was modified by adding MOPS buffer, supplemented with 11 mg/L catechol, 4.6 mg/L thiamin, 11.3 mg/L calcium pantothenate, 5.7 mg/L nicotinic acid, 1.3 mg/L biotin (Wang et al., 2014). For comparisons, the CGXII medium containing 30 mg/L protocatechuic acid (iron chelator) was prepared as well (Unthan et al., 2014).

### Cultivation conditions and adaptive evolution

In general, for growth experiments, cells from frozen glycerol stock cultures were plated on BHI agar and incubated overnight at 30°C. Single colonies were inoculated into 20 ml minimal medium in a 250-ml shake flask and grown for 15-20 h to obtain the pre-culture. The main cultivation was performed in 50 ml minimal medium in a 500-ml shake flask for 24 h. All shake flask cultivations were carried out at 30°C and 220 rpm in a rotary shaker and in biological triplicates. Cell growth was monitored by measuring the optical density at 600 nm ( $OD_{600}$ ) of the culture broth with a UV1800 spectrophotometer (Shimadzu). The dry cell weight was calculated according to the following formula:  $DCW(g/L) = OD_{600} \times 0.3$  (Shinfuku et al., 2009). The measured correlations between DCW and  $OD_{600}$  for different strains are shown in Fig. S7.

The adaptive laboratory evolution (ALE) was carried out in 150 mL shake flasks containing 15 mL minimal medium with 1% glucose at 220 rpm and 30°C. The biomass ( $OD_{600}$ ) was measured every 24 hrs and 150 µl of the culture was transferred into a fresh medium. This process was continued for about 7 months (approximately 1500 generations). Once a week, a culture sample was withdrawn and stored at -80°C for future analysis. Isolates were subsequently obtained from these frozen stocks at week 10 (approximately ~ 500 generations), week 20 (approximately ~ 1000 generations), and week 30 (approximately ~ 1500 generations), respectively, by plating on solid medium.

For the resting cells experiments (oxygen deprivation), 100 ml of over-night cultures (BHI supplemented with 1% glucose) were harvested by centrifugation at room temperature (6000 rpm, 5 min). The cell pellets

were washed twice with 0.9% NaCl after which an appropriate amount of washed cells were re-suspended in 100-ml of MM with 2% glucose in bottles filled to the rim. The cell density obtained corresponded to an OD<sub>600</sub> of approximately 20. Cell suspensions were incubated at 30°C under static conditions with slow magnetic stirring (no oxygen could enter). To prevent a drop in pH, the medium was supplemented 200 mM of MOPS buffer.

### Recombinant DNA techniques and strain construction

All molecular manipulations including PCR amplification, purification, enzymatic digestion, ligation, preparation of competent cells and electroporation were performed according to standard protocols (Sambrook J, Fritsch EF, 1989). All primers used in the current study are listed in Supplementary Table 2. For the re-construction of *ramA*<sup>mut</sup> in the WT or ΔCGP3 strain kindly donated by Prof. Frunzke (Baumgart et al., 2013), the plasmid pK18-*ramA*<sup>mut</sup> was transferred by electroporation into the competent cells and the transformation mixture was plated on BHI agar containing 25 µg/ml kanamycin. After a two-step double homologous recombination event (Becker et al., 2011), colonies being both kanamycin-sensitive and sucrose-resistant were isolated and verified by Sanger sequencing (Macrogen, Korea) to possess the particular mutation. The plasmid pK18-*ramA*<sup>mut</sup> contained two fragments, each approximately 750-bp in size, corresponding to the upstream and downstream regions of the single nucleotide variation (*cg2831*: 155C > T) respectively. The two PCR fragments were amplified and inserted into the empty vector pK18*mobsacB*, which had been digested using *Sma*I according to the Gibson Assembly procedure (Gibson et al., 2009). The assembled mixture was then transformed into *E. coli* TOP10, and the resulting plasmid was designated as pK18-*ramA*<sup>mut</sup>. Similarly, the plasmids pK18-*gntR1*<sup>mut</sup> (*cg2783*: 208G > A), pK18-Δ*gntR1*, and pK18-Δ*ramA* were constructed in the same way, while other re-constructed mutants such as *gntR1*<sup>mut</sup>, Δ*gntR1*, Δ*ramA*, and DKI (*gntR1*<sup>mut</sup> *ramA*<sup>mut</sup>) were created using the plasmids mentioned above respectively.

### Sample preparation and substrate quantification



For isolation of chromosomal DNA from *C. glutamicum*, 2 ml of an exponentially growing culture (OD<sub>600</sub> around 1, BHI medium) were harvested by centrifugation. The cell pellet was re-suspended in 500 µl 0.9% NaCl and treated for two hours, at 37°C, with 30 µl Lysozyme (5 mg/ml) (Sigma Aldrich), after which 15 µl proteinase K (1mg/ml) was added and the incubation was extended for an additional two hours. Afterwards, the chromosomal DNA was extracted as described by Katsumata (Katsumata et al., 1984).

For isolation of RNA from *C. glutamicum*, exponentially growing cells (OD<sub>600</sub> around 1) in minimal medium with 1% glucose were harvested and centrifuged (7000\*g, 5 min at 4°C). The cell pellet from 25 ml culture was washed twice in 0.9% NaCl and then resuspended in 200 µl Solution I (0.3 M sucrose and 10 mM NaAc, pH 4.8) and 200 µl preheated Solution II (2% SDS and 0.01 M NaAc, pH 4.8). Next, RNA was extracted from the homogenized cell lysate using the phenol/acetate/chloroform method described previously (Chan et al., 2014). Finally, the precipitated RNA was dissolved in DEPC-treated water and stored at -80°C.

For intracellular ATP and ADP measurements, 600 µl of exponentially growing culture (MM with 1% glucose) was withdrawn at an OD<sub>600</sub> around 1 and immediately vortexed with same volume of 80°C phenol and glass beads (106 µm in diameter, Sigma). Next, the cell extract was prepared as previously described (Koeblmann et al., 2002b). The concentrations of ATP and ADP were then measured using the luciferin-luciferase ATP Determination Kit (A22066, Thermo Fisher Scientific), as described previously (Liu et al., 2016b).

For determining fermentation products and various sugars an Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column (Bio-Rad, USA) and a Shodex RI-101 detector (Showa Denko KK, Tokyo, Japan) was used. The column oven temperature was set to 60°C, and the mobile phase was 5 mM sulfuric acid with a flow rate of 0.5 ml/min.

### **Whole genome re-sequencing**

Genome re-sequencing was carried out by BGI-Tech (HongKong), using Illumina Hiseq-4000 equipment, and on average, more than 120-fold coverage of the genome was achieved. The resulting data were 100-bp paired end reads, which were trimmed and assembled using Bowtie2 developed by the Langmead Lab (Langmead and Salzberg, 2012). The *C. glutamicum* ATCC 13032 (GenBank accession number:

NC\_006958) genome was used as reference. Variants (SNPs, short indels, and large deletions) were called based on the Bowtie2 assembled data using GATK toolkit by Broad Institute (DePristo et al., 2011). The identified variants were verified by using conventional sequencing.

### Transcriptomics analysis

The transcriptomics analysis was accomplished using Illumina HiSeq-4000 based on the service of RNA-Seq Quantification library at BGI-Tech (HongKong). Datasets consisted of 10M reads per sample with a 50-bp read length. Afterwards, the RNA-seq data was assembled and analyzed by comparing with the translational region of annotated DNA sequence data of reference (GCA\_000196335.1.29) using HiSat2 (Kim et al., 2015) and HTSeq (Anders et al., 2015). The estimation of fold change and other statistical analysis was performed using the DESeq2 package in R (Love et al., 2014). The GO enrichment analysis was carried out by using the function *enrichKEGG* in the clusterProfiler (R-package) (Yu et al., 2012).

### <sup>13</sup>C-metabolic flux analysis

To quantify the intracellular metabolic fluxes, <sup>13</sup>C-MFA was performed. The WT and DKI strains were grown in MM medium with 0.5% glucose (80% 1-<sup>13</sup>C and 20% U-<sup>13</sup>C) in duplicates. The samples were prepared based on the protocol by Zamboni et al. (2009) and the parameters for GC-MS measurements can be seen in Supplementary Table 3. Here we assumed the cellular composition was stable so that any potential adaptations in anabolic fluxes that might have occurred were neglected in the study.

The metabolic flux model used for <sup>13</sup>C-MFA is based on the *C. glutamicum* model described previously and the simulations were performed using OpenFLUX2 software (Shupletsov et al., 2014). The absolute fluxes in mmol/gDW/h are calculated by multiplication with the specific glucose uptake rate (Kromer et al. 2004). At convergence, accurate 95% confidence intervals were computed for most estimated fluxes by evaluating the sensitivity of the minimized SSR to flux variations. Precision or standard deviations (SD) of the estimated fluxes were determined by the following equation:  $SD = (Flux_{upper\ bound\ 95\%} - Flux_{lower\ bound\ 95\%})/4$ .

## Results

### Experimental adaption of *C. glutamicum* in minimal glucose medium

It remains interesting to elucidate the limiting factors that control the specific growth rate of a microorganism, in particular the ones that have a high industrial relevance. In this study, we have tried to address this question for *C. glutamicum* by using ALE. The wild-type strain *C. glutamicum* ATCC13032 was grown for an extended period of time, 7 months, in a defined minimal medium containing glucose. From culture samples obtained after 500, 1,000 and 1,500 generations of growth we isolated colonies which were designated as G5H, G10H, and G15H (Fig.1). Characterization revealed that all isolates grew faster than the wild-type strain (WT) by 31, 37, and 42% respectively, and the faster growth was found to correlate to a similar increase in specific glucose consumption rate ( $6.73 \pm 0.35 \sim 6.87 \pm 0.24$  mmol/g-DCW/h or 33% ~ 37% increase). The biomass yield on glucose and final cell density (OD600) remained unaffected for all strains.

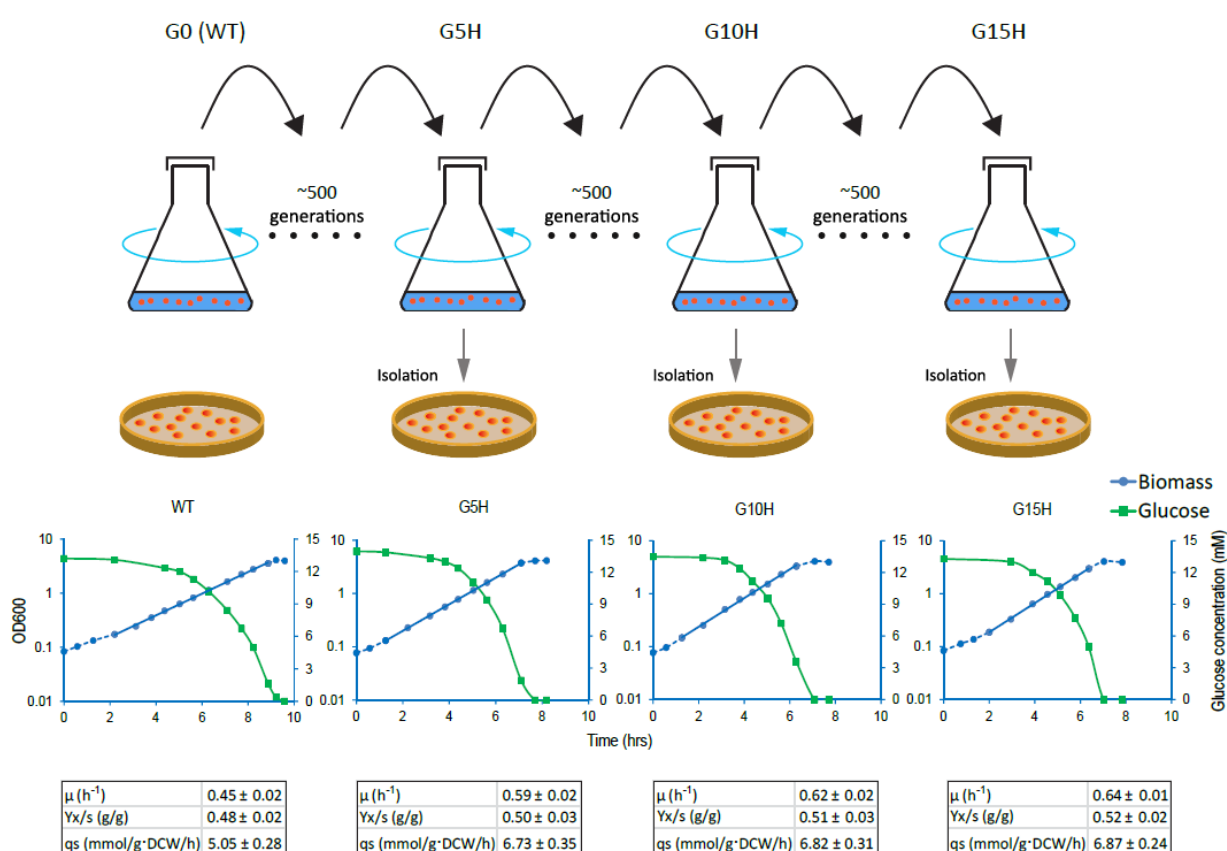


Fig.1 Schematic showing the procedure used for ALE and its outcome. Serial propagation in shaking flask leads to selection of mutants with increased growth rates. Strains were isolated every 500 generations and designated as G5H (500 gen), G10H (1,000 gen), and G15H (1,500 gen). Physiological characterization was performed in biological triplicates and the presented data  $\mu$  (specific growth rate),  $Y_{x/s}$  (biomass yield), and  $q_s$  (glucose consumption rate), were averages based on these independent experiments.

### Whole genome re-sequencing of the isolated adaptive mutants

To identify the mutations responsible for the faster growth phenotype, we sequenced the genomes of the WT, G5H, G10H, and G15H. As shown in Supplementary Fig. S1, all three adapted mutants were found to lack a 180 kbp genomic fragment (*cg1890* – *cg2065*), which previously has been identified as the prophage CGP3 cluster (*cg1890* - *cg2071*) (Baumgart et al., 2013; Kalinowski et al., 2003).

Although the end part of the CGP3 cluster was conserved in our mutants, dozens of mutations were observed in the genes *cg2066* and *cg2069*. Aside from mutations in the prophage region or in the intergenic region (*cg1501/1502*, *cg1562/1563*, *cg1889/1890*), only four additional mutations were found, and these were shared between the three isolates: C to A exchange at position 83 in *cg0712* (hypothetical protein), leading to amino acid replacement of Thr-28 by Asn; G to A exchange at position 208 in *cg2783* (GntR1: GntR family transcriptional regulator), leading to amino acid replacement of Glu-70 by Lys; C to T exchange at position 155 in *cg2831* (RamA: LuxR family transcriptional regulator), leading to amino acid replacement of Ala-52 by Val; A to G exchange at position 878 in *cg3165* (hypothetical protein), leading to amino acid replacement of Asp-293 by Gly (Fig. 2). A summary of the genomic re-sequencing results can also be seen in Supplementary Data 1.

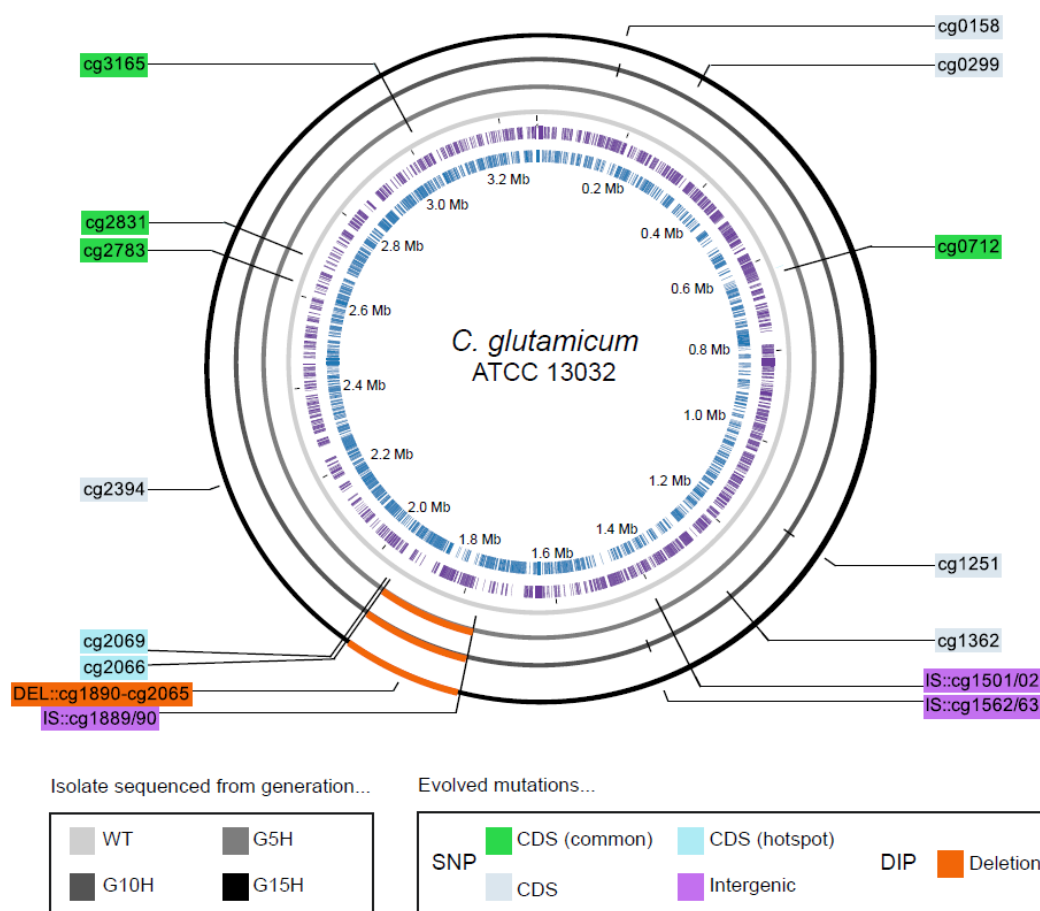


Fig. 2 Mutations found by sequencing the three faster growing mutants obtained in the ALE experiment. The four rings, from outer to inner, represent the genomes of G15H, G10H and G5H sampled at 1,500, 1,000, and 500 generations respectively, and that of the WT. The innermost circle (blue) indicates the genome position in megabase pairs (Mb). Also indicated are the SNP's, which are highlighted in either green, light blue, light grey when they occur in genes or purple when located in intergenic regions. Noticeably, the light blue highlighting indicates many mutations in genes *cg2066* and *cg2069* (hotspots). The region indicated in orange corresponds to the prophage region (CGP3), which is missing in all three ALE strains. SNP: single nucleotide polymorphism; DIP: deletion/insertion polymorphism.

### Identifying the causative mutations.

To confirm the involvement of the four common mutations discovered above, we first introduced each of them in the WT background. Only the mutation in *ramA* seemed to have an effect, however, the effect was a noticeable 20% increase in specific growth rate (Supplementary Fig. S2). Previously it has been reported that when the prophage region is deleted ( $\Delta$ CGP3), the cell phenotype is insignificantly affected (Baumgart et al.,

2013; Unthan et al., 2015). Since all the faster growing mutants had a large truncation and several mutations in this region, we decided to assess the effect of the four mutations in a  $\Delta$ CGP3 strain background.

Consistent with previous findings, the  $\Delta$ CGP3 strain grew as fast as the WT on glucose. Surprisingly, in the  $\Delta$ CGP3 background, both the *ramA* (LUXR:  $\Delta$ CGP3 with *ramA*-A52V) and the *gntR1* mutation (GNTR:  $\Delta$ CGP3 with *gntR1*-E70K) had positive effects on growth. The growth rate was increased by 22% due to the *ramA* mutation ( $0.55 \pm 0.02 \text{ h}^{-1}$ ) and by 8.8% ( $0.48 \pm 0.01 \text{ h}^{-1}$ ) (Fig. 3A) when the *gntR1* mutation was introduced. We further found that the effects of the single mutations were additive, and the double knock-in strain, DKI, grew almost as fast ( $0.62 \pm 0.02 \text{ h}^{-1}$ ) as the adapted mutants. The increase in specific growth rate also correlated well with an equally large increase in glucose consumption rate, 36% higher than that of the WT ( $6.92 \pm 0.32 \text{ mmol/g}\cdot\text{DCW/h}$ ). More details can be seen in Supplementary Table 4.

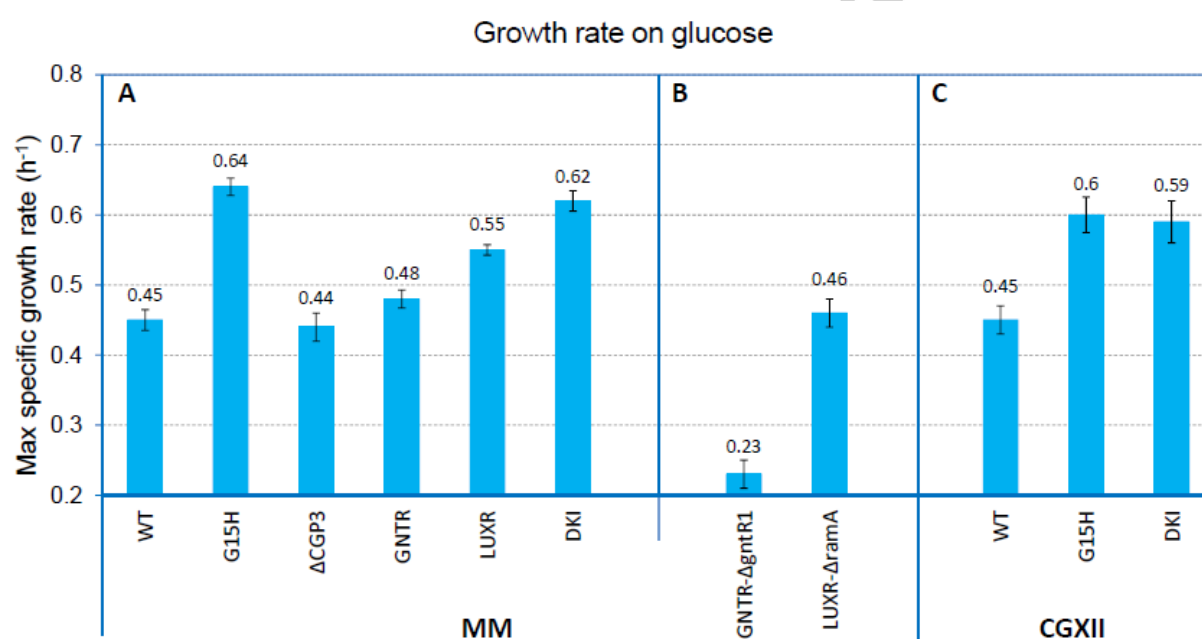


Fig. 3 A comparison of the specific growth rates of adapted or reconstructed mutants on glucose. A: Specific growth rates on MM for the following strains are shown: G15H (mutant isolated in ALE experiment after 1,500 generations);  $\Delta$ CGP3 (the WT lacking the CGP3 region); GNTR ( $\Delta$ CGP3 with *gntR1* mutant); LUXR ( $\Delta$ CGP3 with *ramA* mutant); DKI ( $\Delta$ CGP3 with the *gntR1* and *ramA* mutations). B: Specific growth rates on MM for GNTR- $\Delta$ gntR1 and LUXR- $\Delta$ ramA were determined as well. C: As a complementary test, the growth rates of WT, G15H, and DKI strain are also compared on the more common defined medium CGXII (Unthan et al., 2014). The growth rates and standard deviations were calculated based on data from three independent experiments.

It has previously been found that a *ramA* (*cg2831*) knock-out mutant is unable to grow on acetate as sole carbon source, but that it grows just as well as the WT on glucose (Cramer A, et al., 2006). We found that the LUXR strain, which carries a mutated *ramA*-A52V gene, is able to grow on acetate (Supplementary Fig. S3), and this indicates that the mutation does not lead to inactivation but rather changed function. To prove this, the mutated *ramA*-A52V gene was deleted from LUXR. The resulting strain, LUXR- $\Delta ramA$ , indeed was neither able to grow on acetate, nor display improved growth on glucose (Supplementary Fig. S3 and Fig. 3B). To assess whether the same could be the case for the mutation in *gntR1* in the GNTR strain, we deleted this gene and found that the growth rate of the GNTR- $\Delta gntR1$  mutant on glucose was reduced by approximately 50%, when compared to the GNTR strain (Fig. 3B). It has been shown previously that there are two functionally redundant GntR-type regulators encoded by *gntR1* and *gntR2* in *C. glutamicum*, and a mutant lacking both *gntR1* and *gntR2* has a 60% reduction in glucose uptake and specific growth rate when compared to the WT (Frunzke et al., 2008). Since *gntR2* is located in the CGP3 region, which is missing in the adapted strains (and in the  $\Delta CGP3$  strain), the constructed GNTR- $\Delta gntR1$  mutant essentially would be identical to a  $\Delta gntR1 \Delta gntR2$  mutant, which explains the similar phenotypes.

In the current study we used a minimal medium differing slightly from the more commonly used CGXII medium (Unthan et al., 2014), and for this reason we decided to test the strains in this medium as well. As expected, both the G15H and the DKI strain displayed comparable high specific growth rates, which were approximately 33% higher than that of the WT (Fig. 3C).

### **The *gntR1* and *ramA* mutations improve the metabolic efficiency**

The above characterization revealed the involvement of the *gntR1* and *ramA* mutations in the faster growth phenotype, and this improvement was not caused by inactivation of either RamA or GntR1. To gain additional information, we decided to look into the global gene expression profile of the different strains. In Fig. 4A, we provided a simplified overview of the results obtained from an analysis of the transcriptome profiles for the WT, G15H, LUXR, and DKI strains. The hierarchical clustering results revealed that the

global gene expression profile of the adapted mutant G15H was similar to that of the double knock-in mutant DKI, however it was quite different from that of the WT strain. By applying KEGG enrichment analysis to interrogate the functions of differentially expressed genes (Fig. 4B) we could see that the genes that were differentially expressed in DKI and G15H were involved in cellular processes such as “microbial metabolism in diverse environments”, carbon metabolism, glycolysis, and the pentose phosphate pathway (PPP), i.e. all growth-associated categories. The LUXR strain exhibited a less pronounced differential gene expression profile, and fewer cellular processes seemed to be affected when compared to that of G15H or DKI, which correlated well with the smaller effect on growth rate. Moreover, we compared the expression level of 33 genes involved in the central metabolism for the four tested strains (Fig. 4C) and found that most of the genes involved in glycolysis were significantly upregulated in LUXR and DKI, which could indicate that the mutated RamA has changed its function, to become a global up-regulator of glycolytic genes. The PPP genes were greatly upregulated in both DKI and G15H, which indicates an involvement of the mutated GntR. On the contrary, some TCA genes especially for *sucBD* were down-regulated in the fast growing cells. This may demonstrate the transcriptional control of TCA flux is low in *C. glutamicum*. In addition, the anaplerotic reactions encoded by *ppc* were up-regulated, while the reverse reaction driven by *pck* was significantly down-regulated. According to a previous study, deletion of the prophage CGP3 region leads to a strong reduction in the expression of *pck* (Baumgart et al., 2013). More detailed information regarding the transcriptomics analysis is provided in Supplementary Data 2.



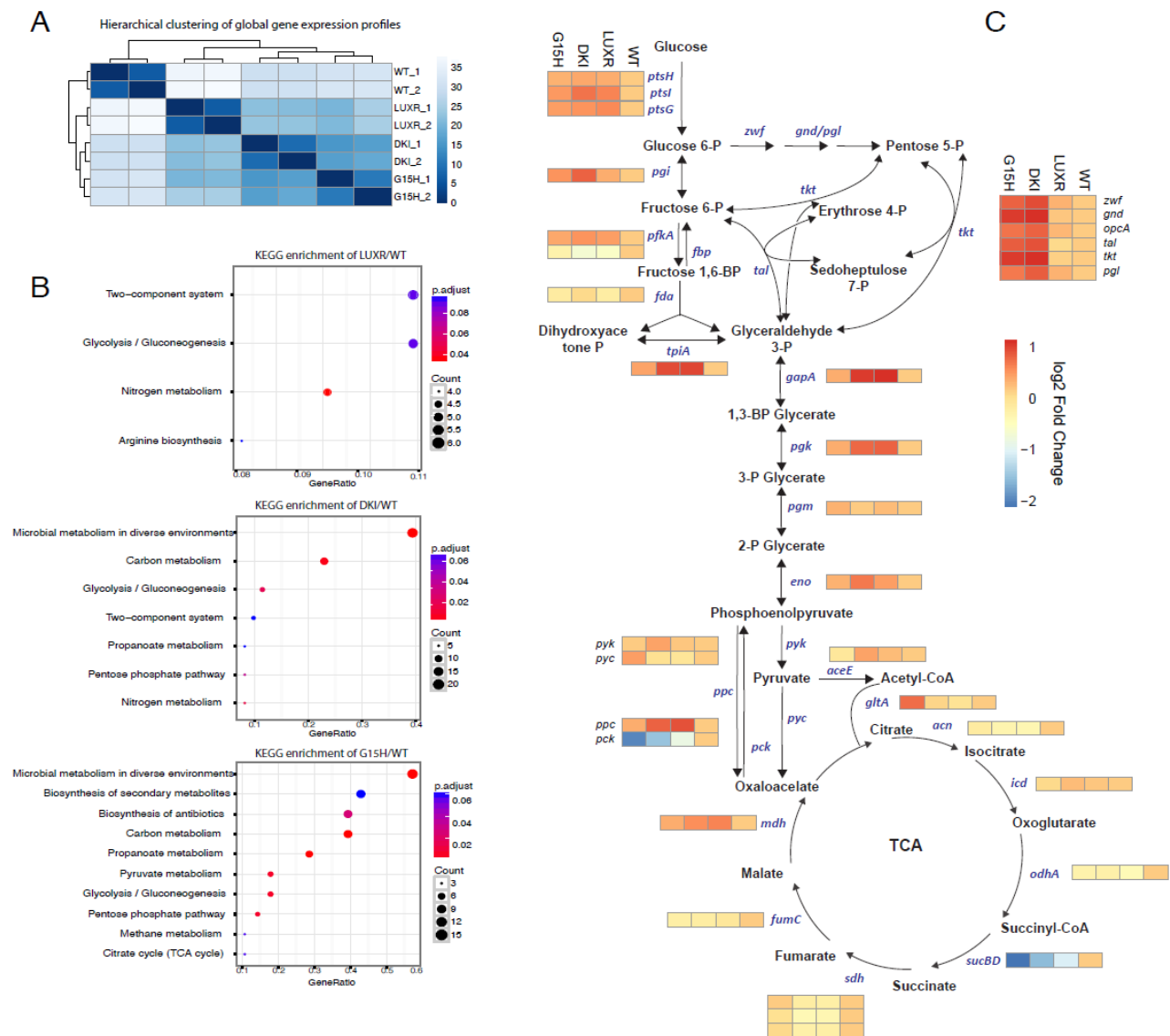


Fig. 4 Comparison of global gene expression and contextualization of gene expression data in the central metabolic network. (A) Hierarchical clustering of gene expression profiles for four tested strains after normalization using DESeq2. The transcriptomics data for each strain was obtained based on biological duplicates. (B) The genes that are differentially expressed among the reconstructed mutants and WT have been classified according to gene functional annotations by using KEGG enrichment analysis. The color of circles (from purple to red) represents the adjusted p-value from higher to lower, while the size of circles represents the number of qualified gene counts. (C) The expression of a series of genes involved in central metabolism is compared using a heat map. The strains from left to right are: G15H, DK1, LUXR, and WT respectively.

For the DK1 and G15H strains the *gntP* (encoding gluconate permease) and *gntK* (encoding gluconate kinase) were strongly upregulated, to a level around 9- and 20-fold higher than in the WT strain, respectively

(Supplementary Data 2). This could indicate a potential role in the fast growth phenotype, however, deletion of either gene did not have any effect on growth (Supplementary Fig. S4).

In addition, the apparent growth rate limitation on glucose, for the wild-type, could be due to either a limitation in catabolism or anabolism. In the latter case it is commonly found that the intracellular energy charge, or more simply the ATP/ADP ratio, is high, whereas a limitation in catabolism can lead to a low ATP/ADP ratio (Koeblmann et al., 2002b). We measured the intracellular ATP and ADP concentration of the WT, DKI, and G15H, and found that the ATP/ADP ratios for DKI and G15H were quite comparable, and around 25% higher than that of the WT (Supplementary Fig. S5). This may indicate the presence of a limitation in catabolism in the WT strain, which the transcriptomics analysis also supported.

### **<sup>13</sup>C metabolic flux analysis**

Changes in transcription levels of genes involved in the central carbon metabolism can indicate metabolic shifts. To determine whether the high growth rate could be associated with changes in the metabolic flux redistribution, we performed <sup>13</sup>C-MFA for the wild type and DKI strains. The results are shown in Fig. S6, where the fluxes shown have been normalized to 100 units of glucose uptake (80% 1-<sup>13</sup>C and 20% U-<sup>13</sup>C). We found that the relative metabolic fluxes in glycolysis and TCA cycle were remarkably similar for the two strains, but a small increase in the PPP flux was still found for the DKI strain. The raw measured and fitted labelling data can be found in Supplementary data 3. In absolute terms, as the glucose uptake rates were significantly elevated from 5.05 mmol/g DCW/h in the wild type to 6.92 mmol/g DCW/h in DKI, the overall metabolic rates were increased as shown in Fig. 5. These data show that, even though, the genes in glycolysis were broadly upregulated and the genes in TCA cycle were downregulated, this did not correspond to a significant change in the metabolic flux distribution. Our results are consistent with the recent findings of Long et al., for evolved *E. coli* strains, where the intracellular metabolic fluxes changed very little after ALE, although the growth rate had been increased by up to 1.6-fold (Long et al., 2017). They found that the faster growth of adaptively evolved strains was not due to an intracellular flux redistribution, but rather caused by increases in glucose uptake rate and an overall boosted central metabolism. In the

current flux analysis model, we considered the cellular composition to be stable so that it could not cover all potential adaptations in anabolic fluxes that might have occurred.

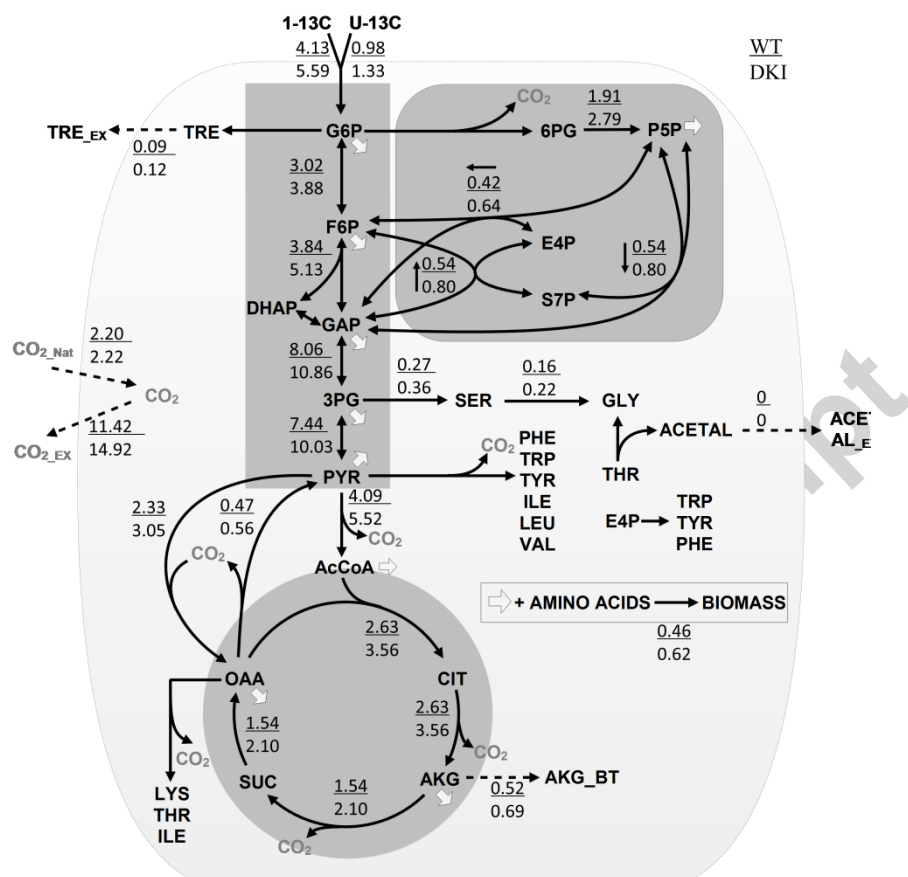


Fig. 5 Metabolic flux analysis of the central carbon metabolism in the wild type (WT, upper values) and DKI (lower values). All the fluxes are given in mmol/g DCW/h. The glucose uptake rates were 5.05 mmol/g DCW/h in the wild type and 6.92 mmol/g DCW/h in DKI, respectively. Abbreviates: 1-13C, 1-<sup>13</sup>C glucose. U-13C, U-<sup>13</sup>C glucose. G6P, glucose-6-phosphate. F6P, fructose-6-phosphate. GAP, glyceraldehyde 3-P. DHAP, dihydroxyacetone P. 3PG, 3-P glycerate. PYR, pyruvate. TRE, trehalose. 6PG, 6-phosphogluconate. P5P, pentose 5-P. E4P, erythrose 4-P. S7P, sedoheptulose 7-P. CIT, citrate. AKG, a-ketoglutarate. SUC, succinate. OAA, oxaloacetate.

**The effect of the *gntR1* and *ramA* mutations on growth is not restricted to glucose.**

We decided to investigate whether the beneficial effects on growth, conferred by the *gntR1* and *ramA* mutations, could be observed on other carbon sources besides glucose. As shown in Fig. 6, both the G15H and DKI strains were found to grow faster on sucrose and fructose, G15H by approximately 20% ( $\mu_{G15H,Suc} = 0.67 \pm 0.02 \text{ h}^{-1}$ ;  $\mu_{G15H,Fru} = 0.61 \pm 0.01 \text{ h}^{-1}$ ) and the DKI strain by approximately 15% ( $\mu_{DKI,Suc} = 0.64 \pm 0.01 \text{ h}^{-1}$ ;  $\mu_{DKI,Fru} = 0.59 \pm 0.01 \text{ h}^{-1}$ ) when compared to the WT ( $\mu_{WT,Suc} = 0.56 \pm 0.02 \text{ h}^{-1}$ ;  $\mu_{WT,Fru} = 0.51 \pm 0.02 \text{ h}^{-1}$ ). Similar to the phenotype on glucose, here we also tested the growth rate of the  $\Delta$ CGP3 strain on sucrose and fructose, however, the strain grew similar to that of the WT (Figure not shown). In addition, the plasmid pXYL, a derivative of pECX99E containing *xylA* and *xylB* gene from *Lactococcus lactis* KF147 for xylose metabolism, was transformed into the WT and DKI strain as well. The latter strain ( $\mu_{DKI-pXYL,Xyl} = 0.18 \pm 0.01 \text{ h}^{-1}$ ) also exhibited a 37% higher growth rate compared to the WT-pXYL strain ( $\mu_{WT-pXYL,Xyl} = 0.13 \pm 0.02 \text{ h}^{-1}$ ) when growing on xylose as the sole carbon source. In contrast, on the gluconeogenic carbon sources lactate and acetate, the three strains performed very similarly (Fig. 6). Also, the stimulatory effect of the mutations could only be seen on minimal medium, and not when the strains were grown in rich BHI medium.

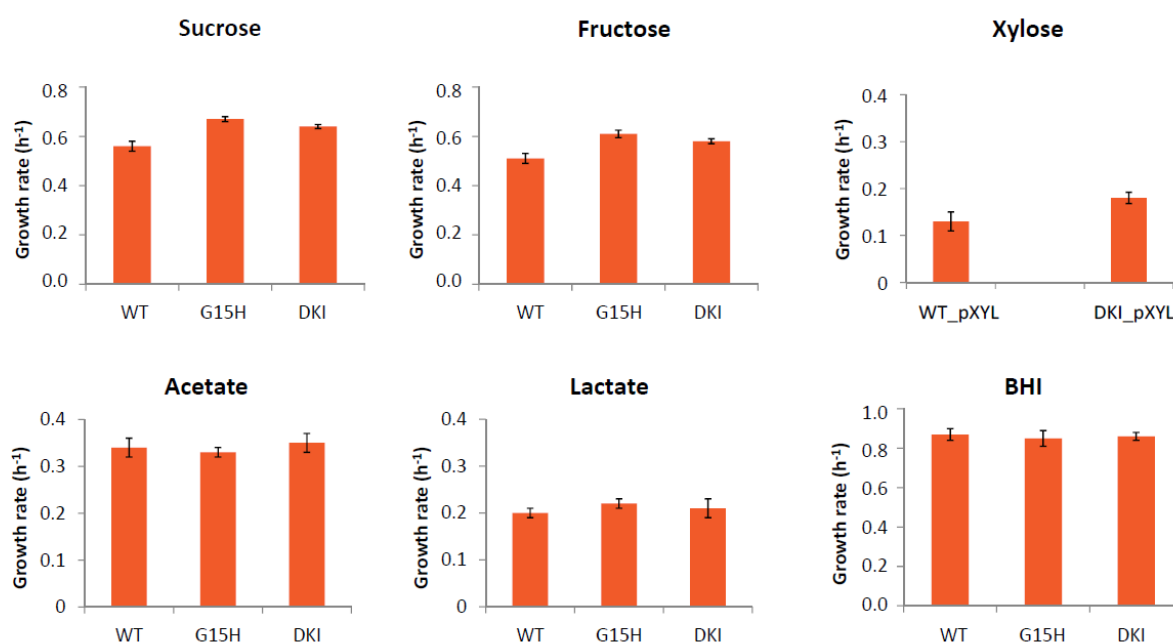


Fig. 6 Comparison of growth rates for the WT, G15H, and DKI strain growing on different carbon sources. The concentration of sucrose and fructose was 10 g/L, whereas the concentration of lactate and acetate was 150 mM. BHI (Brain heart infusion, Sigma) is a rich medium supporting fast growth. The specific growth rates are calculated based on data from three biological replicates.

### **The *gntR1* and *ramA* mutations enhance glycolytic capacity under anaerobic conditions**

Several interesting studies have shown the potential of using *C. glutamicum* as a whole cell catalyst under anaerobic conditions where the cells are unable to grow (Inui et al., 2004; Michel et al., 2015). Both the *ramA* and the *gntR1* mutations had a positive effect on the glycolytic flux when the cells were growing, and for this reason, we decided to investigate whether the mutations also could have an effect under anaerobic conditions. The different strains were first grown in rich medium, harvested and then re-suspended to an  $OD_{600} \approx 20$  in minimal medium with 2% glucose, after which glucose consumption and lactate production rates were compared (at 30°C). Since lactate is formed under these conditions, the medium was supplemented with 200 mM MOPS serving as a pH buffer. As shown in Fig. 7, G15H and DKI consumed glucose at comparable rates ( $q_{G15H,glu} = 2.75 \pm 0.18$  mmol/g-DCW/h;  $q_{DKI,glu} = 2.71 \pm 0.22$  mmol/g-DCW/h) and lactate formation rates were also quite similar for the two strains ( $q_{G15H,lac} = 3.67 \pm 0.27$  mmol/g-DCW/h;  $q_{DKI,lac} = 3.62 \pm 0.22$  mmol/g-DCW/h). When compared to the WT these fluxes were approximately 30% higher than that of the WT ( $q_{WT,glu} = 2.09 \pm 0.16$  mmol/g-DCW/h;  $q_{WT,lac} = 2.81 \pm 0.23$  mmol/g-DCW/h). In a previous study it was shown that, by over-expressing several glycolytic genes (*pgi*, *pfk*, *gapA*, and *pyk*), the anaerobic glucose metabolism and alanine production (Yamamoto et al., 2012) could be improved. Our results reveal a more simple way to achieve the similar effect, i.e. introducing mutations in *gntR1* and *ramA*. However, the previous study was unable to stimulate the glycolytic flux in growing cells as was accomplished in our study.

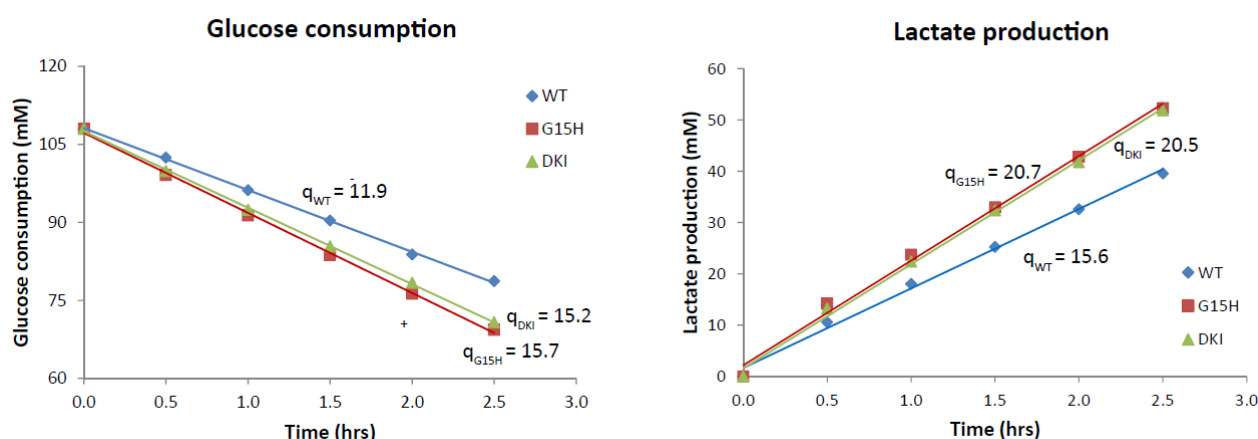


Fig. 7 Glucose consumption and lactate formation rates for WT, G15H, and DKI under anaerobic conditions. Cells grown in rich BHI medium were re-suspended in minimal medium with 2% glucose and 200 mM MOPS buffer to an  $OD_{600} \approx 20$ . Data from one representative out of three similar biological replicates is shown.

### The *gntR1* and *ramA* mutations enhance the lysine yield and productivity

Finally, we tested the effects of *gntR1* and *ramA* mutations on lysine production. The two mutations were introduced into a lysine-producing strain (LysC), containing a mutation in *lysC* conferring feedback-resistance ( Thr-311->Ile in *lysC* gene, Ohnishi et al., 2002). As shown in Fig. 8, we compared the performance of LysC and DKI\_LysC strain in MM with 0.5% glucose, and observed that the specific growth indeed was increased from  $0.43 \text{ h}^{-1}$  (LysC) to  $0.59 \text{ h}^{-1}$  (DKI\_LysC), i.e. the same effect as observed for the WT. More interestingly, the lysine titer was also increased 32% for the DKI\_LysC strain. Because of the mutations, the entire fermentation process could be shortened from 11 hrs to 9 hrs (Fig. 8), which corresponds to an average increase in volumetric lysine productivity of 100%. The effect that these two mutations have on the performance of high-yield, high titer industrial lysine-producing strains remains to be seen.

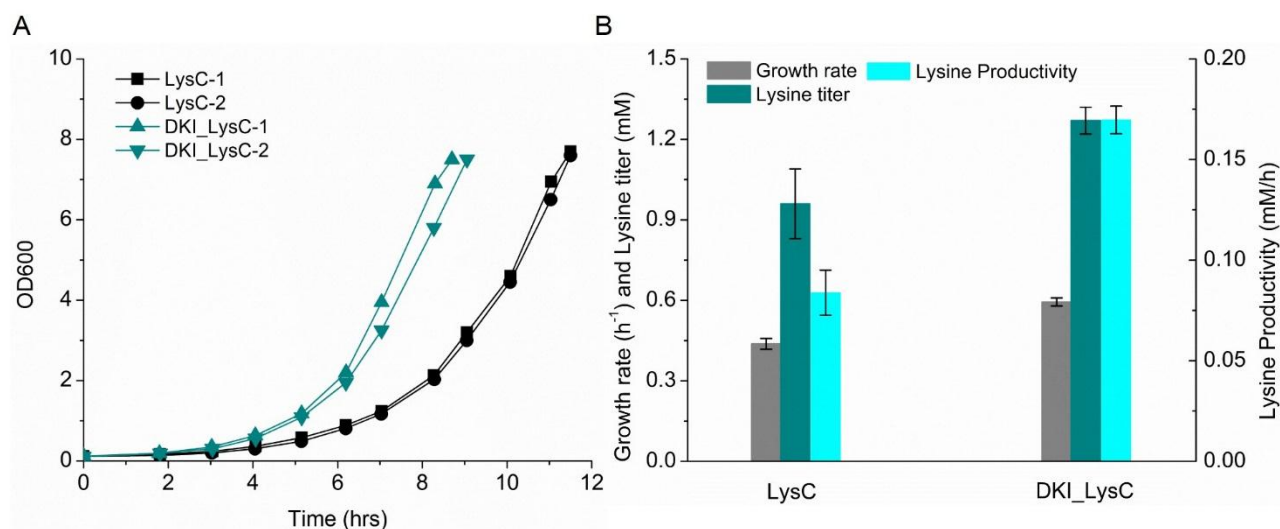


Fig. 8 Characterization for the LysC and DKI\_LysC strain. Cells were cultivated in the minimum medium with 5 g/L glucose. A. the growth. B. the specific growth rate, lysine titer and lysine productivity. The growth characterization was performed by biological duplicates.

## Discussion

As mentioned above, the specific growth rate is an important parameter for microorganisms with industrial relevance, such as *C. glutamicum*. *C. glutamicum* grows well in cheap defined media, albeit with a somewhat low specific growth rate (0.45 h<sup>-1</sup>) and few studies have focused on what limits the specific growth rate for this particular organism (Keilhauer et al., 1993; Liebl et al., 1989). Recently, Noack and his colleagues reported a high specific growth rate of 0.61 h<sup>-1</sup> for *C. glutamicum* growing in a defined minimal medium containing glucose (Unthan et al., 2014) and protocatechuic acid (PCA). The reason for the fast growth was found to be simultaneous metabolism of glucose and PCA, which enter metabolism at different points. Where glucose is metabolized via glycolysis, PCA in few steps is converted into succinyl-CoA and acetyl-CoA that directly enter the TCA cycle. For industrial applications such co-feeding approaches are only useful when the substrates are cheap, and using PCA is not a viable option due to its high cost. It is therefore relevant to investigate whether the specific growth rate could be increased by other means. Through long-term ALE, we successfully isolated several mutants which grew faster ( $\mu_{\max} \approx 0.64\text{h}^{-1}$ ) than the WT and we

identified the mutations responsible for most of the growth improvement and demonstrated that these SNPs can be stably inherited (Fig. 2).

One important mutation was located in *gntR1* (*cg2783*), encoding GntR1, which previously has been identified as a transcriptional regulator of gluconate catabolism together with GntR2 encoded by *gntR2* (*cg1935*) (Frunzke et al., 2008). These two regulators have been proven to be functionally redundant. It has been demonstrated that the gluconate permease (*gntP*), gluconate kinase (*gntK*), gluconate 6-phosphat dehydrogenase (*gnd*) and genes in the PPP are all strongly repressed by GntR1 and GntR2, while the glucose PTS (*ptsG*) is activated by them. A mutant lacking both regulators metabolizes glucose 60% slower, and therefore also grows quite slowly. However, in this study, we found that *gntR2* had been lost along with the prophage region (CGP3) in the fast growing adapted mutants, and that *gntR1* had been mutated (*gntR1*-E70K), which resulted in an increased expression of *gntP* (10.5-fold) and *gntK* (42.2-fold). Frunzke et al., 2008 characterized a  $\Delta gntR1\Delta gntR2$  mutant (Frunzke et al., 2008) and observed the same effect on expression of these two genes. However, in contrast to what we found for the deletion strain, neither the expression of *ptsG* nor cell growth rate on glucose had been reduced in the *gntR1*-E70K strain, which indicated that the E70K mutation did not inactivate *gntR1* completely. Since the binding motif of *gntP* (TTTGATCATACTAAT) and *gntK* (TATGATAGTACCAAT) only shared little similarity with that of *ptsG* (AAAAGTATTACCTTT), we hypothesize that the effects from the E70K mutation on expression of these genes might be different.

The RamA (*cg2831*) protein is a LuxR-type global transcriptional regulator in *C. glutamicum* and it controls expression of genes involved in sugar uptake, glycolysis, gluconeogenesis, acetate, and several other metabolic pathways (Auchter et al., 2011). It was first identified as a transcriptional activator of genes involved in acetate metabolism by its binding to the promoter region of *pta-ack*, and its inactivation was found to completely abolish growth on acetate (Cramer A et al., 2006). Later it was shown that RamA could serve as a repressor of the glycolytic gene *gapA* (Toyoda et al., 2009) and as a positive regulator of the TCA cycle genes *sdhCAB* (succinate dehydrogenase operon) and *acn* (aconitase) (Bussmann et al., 2009; Teramoto et al., 2011). In total 55 genes appear to be regulated by RamA, of which 45 have been verified experimentally and the remaining candidates have been predicted to be regulated as shown in CoryneRegNet



(<http://www.coryneregnet.de>) (Pauling et al., 2012). However, despite accumulating knowledge regarding the regulatory roles of RamA, there have been no reports describing beneficial effects on central metabolism from mutations in *ramA*. In this study we found such a mutation, which did not result in impairment of acetate metabolism. The transcriptomics data on glucose indicated that the glucose PTS and glycolytic genes were up-regulated to some extent, which correlated nicely with the increased glucose uptake rate and growth rate, but a specific bottleneck responsible for the slower growth of the wild-type could not be pinpointed. Indeed, growth may be regulated in a more complicated manner, and simple overexpression of a “rate-limiting” step might not be sufficient to overcome such regulatory mechanisms. We found that the expression of most TCA cycle genes were slightly decreased except for the succinyl-CoA synthase genes *sucBD*, however, no effects were observed on the TCA cycle flux (Fig. S6).

When this article was under review, a study describing a *C. glutamicum* mutant with a 28% improvement in specific growth rate was published (Pfeifer et al. 2017). Although a similar approach was used to obtain this mutant, fast growth was found to be due to different mutations, e.g. in genes encoding pyruvate kinase and 1-phosphofructokinase (*fruK*). The latter mutation is interesting as we previously have found that a *fruK* and *ptsF* double mutant is able to grow 10% faster in glucose minimal medium (Wang et al., 2016). In the study by Pfeifer et al. growth characterization was carried out in a Biolector, i.e. automated equipment, using a 750 µl fermentation volume. Curiously for the same wild-type strain used in our study, and using the same medium CGXII, they obtained a specific growth rate that was 16% higher than what we achieved using standard shake flasks. It is possible that the small fermentation volume allowed for a more efficient aeration, and that this could somehow explain the effect. Another difference between these two studies is that the large truncations found in the genome of our evolved strains (Fig. S1) did not appear in their evolved strains. This may be due to the different evolution method or medium (MM or CGXII) used. However, both studies clearly demonstrate that there is space to improve the growth fitness of *C. glutamicum*.

In this study we also characterized growth on other carbon sources in addition to glucose. Interestingly, when grown on sucrose and fructose, the G15H and DKI strain also displayed a 15% ~ 20% increase in specific growth rate when compared to the WT strain. This indicates that the glycolytic capacity, in general, has been improved by the *gntR1*-E70K and *ramA*-A52V mutations. Even more surprising was the finding that growth

on xylose could be improved (a strain containing a plasmid expressing *xyxAB* from *L. lactis*) by the *gntR1* and *ramA* mutations, despite the fact that the strain without the mutations grew very slowly, and had a very low glycolytic flux indicating that glycolytic capacity should not be an issue. In addition, it has been shown that the glycolytic flux in non-growing cells is far below that of growing cells, and that this can be explained by the reduced ATP demand in non-growing cells (Koeblmann et al., 2002a). Surprisingly Yamamoto et al., 2012 were able to increase the glycolytic flux in non-growing cells by overexpressing various glycolytic genes, which seems counter-intuitive as a build-up of ATP would be expected to slow down glycolysis. We decided to examine whether our faster growing strains also performed better under such conditions, and indeed we observed an increased glucose uptake and lactate production for the non-growing cells. To elucidate how the mutations in *gntR1* and *ramA* change their function is next in the pipeline, and for this purpose a reliable protein structure model could be useful for predicting the potentially altered regulation or activation site by the single amino acid variation.

## Conclusion

Using adaptive laboratory evolution we have obtained a mutant of *C. glutamicum* with the hitherto fastest growth on glucose minimal medium, and have found the main reason for the fast growth to be two mutated transcriptional regulators *gntR1*-E70K and *ramA*-A52V. The beneficial effect on growth of these mutations is not restricted to glucose alone, and growth on the important industrially relevant carbohydrates sucrose, fructose and xylose was stimulated as well. We thus demonstrate that it is possible to achieve remarkable improvements in performance of this highly important industrial workhorse by minimal manipulation, which is important to avoid unnecessary metabolic perturbations and to maximize the efficiency of *C. glutamicum* based cell factories.

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### Author contributions

P.R.J. and C.S. conceived the project. P.R.J., C.S., and Z.W. designed the research and coordinated the project. Z.W., J. L., and L. C. performed the experimental works and data analysis with the help from A. Z. Z.W prepared the manuscript with the help from J.L, P.R.J, and C.S.

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